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EXHIBIT 1

E. Konadu, et al., "Synthesis, Characterization, and Immunological Properties in Mice of Conjugates Composed of Detoxified Lipopolysaccharide of *Salmonella paratyphi* A Bound to Tetanus Toxoid, with Emphasis on the Role of *O* Acetyls," *Infection and Immunity*, Vol. 64, No. 7, July 1996, pp. 2709-2715.

INFECTION AND IMMUNITY, July 1996, p. 2709–2715
0019-9567/96/$04.00+0

Vol. 64, No. 7

Synthesis, Characterization, and Immunological Properties in Mice of Conjugates Composed of Detoxified Lipopolysaccharide of *Salmonella paratyphi* A Bound to Tetanus Toxoid, with Emphasis on the Role of *O* Acetyls

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Received 30 November 1995/Returned for modification 12 March 1996/Accepted 16 April 1996

***Salmonella paratyphi* A, the second most common cause of enteric fever in Southeast Asia, is a habitant of and a pathogen for humans only. Lipopolysaccharides (LPS) are both essential virulence factors and protective antigens for systemic infections caused by groups A, B, C, and D nontyphoidal salmonellae. The O-specific polysaccharide of *S. paratyphi* A is composed of a trisaccharide, →2-α-D-Man*p*-(1→4)-α-L-Rha*p*-(1→3)-α-D-Gal*p*-(1→, with a branch of D-paratose from the C-3 of α-D-mannose, and the C-3 of β-L-rhamnose is partially O acetylated (C. G. Hellerqvist, B. Lindberg, K. Samuelsson, and A. A. Lindberg, Acta Chem. Scand. 25:955–961, 1971). On the basis of data from our investigational vaccines for enteric bacterial pathogens, including group B salmonellae (D. C. Watson, J. B. Robbins, and S. C. Szu, Infect. Immun. 60:4679–4686, 1992), conjugates composed of the detoxified LPS of *S. paratyphi* A bound to tetanus toxoid (TT) were prepared by several schemes. LPS was detoxified with acetic acid or with hydrazine; the latter removed *O* acetyls from the O-specific polysaccharide. The detoxified polysaccharides were activated with cyanogen bromide (CNBr) or with 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) and bound to TT with or without a spacer. Solutions of 2.5 μg of saccharide, alone or as a conjugate, were injected subcutaneously into young mice, and LPS and TT antibodies were measured by enzyme-linked immunosorbent assaying. A conjugate synthesized with higher-molecular-weight O-SP elicited the highest anti-LPS levels. Only conjugates with *O* acetyls elicited serum immunoglobulin G anti-LPS with bactericidal activity. There were no statistically significant differences between LPS antibody levels elicited by conjugates synthesized with or without a spacer. The conjugate with O-specific polysaccharide activated by CDAP and bound to TT without a spacer elicited the highest level of TT antibodies. Clinical evaluation of *S. paratyphi* A conjugates is planned.**

Salmonellosis remains a common and serious disease. Recently, the incidence of infection with nontyphoidal salmonellae in the United States has risen (9, 14, 28). *Salmonella* infection can cause enteritis that may be complicated by bacteremia (enteric fever) and extraintestinal complications in normal and especially in immunocompromised subjects. The highest incidence, morbidity, and mortality of infections caused by salmonellae are in children (48, 56). Children in developing countries and patients with sickle cell disease or AIDS are particularly susceptible to enteric fever and its extraintestinal complications (3, 13, 19, 41, 43–46, 48, 52, 73, 75). Treatment of enteric fever, including *Salmonella paratyphi* A, has become more difficult with the emergence of multiantibiotic-resistant strains (4, 7, 8, 27, 31, 39, 40, 43, 45, 53, 69, 71).

There are no licensed vaccines for nontyphoidal salmonellae. Although used by many countries for decades, TAB vaccine, which is composed of inactivated cells of *Salmonella typhi* and groups A and B salmonellae, was removed as a licensed product because efficacy was demonstrable only for the T component (typhoid fever). The lipopolysaccharide (LPS) of salmonellae is both a virulence factor and a protective antigen. Although there are approximately 50 LPS serogroups, almost all extraintestinal infections in humans (enteric fever) are caused by serogroups A, B, C_1, C_2, and D (3, 9, 20, 43–46, 48, 73). In Southeast Asia, as in developing countries, the most common cause of enteric fever is *S. typhi* (1, 2, 7, 31, 39, 54). The second most common cause in this region is *S. paratyphi* A, which accounts for approximately 15% of cases (1, 4, 22, 27, 33, 47, 60, 62, 71); although widespread after World War II (20, 33, 54, 64, 76), at present this serogroup is rarely found in other parts of the world (8, 28, 59). *S. paratyphi* A is similar to *S. typhi* in several ways. (i) Both are inhabitants and pathogens for humans only (25, 56). (ii) Both can be considered clones (21, 35, 49, 55, 59). With the exception of strains from Indonesia (24), there is only one serotype of *S. typhi* (O9, 12:Vi:d). Similarly, there is only one serotype of *S. paratyphi* A (O1, 2, 12:a:−) (21, 35, 59). A variant lacking factor 1 (*S. paratyphi* var. durazzo [35]) was reported. (iii) The pathogeneses of enteric fever caused by *S. typhi* and nontyphoidal salmonella, including *S. paratyphi* A, are similar. Both pathogens are ingested and may enter the bloodstream. The surface polysaccharide of *S. typhi* is the Vi capsular polysaccharide (Vi), and serum antibodies to this antigen confer immunity to typhoid fever (2, 36, 56–58). The surface polysaccharide of *S. paratyphi* A is the O-specific polysaccharide of its LPS. On the basis of studies of the structurally related serogroup B (*Salmonella typhimurium*) (10–12, 16, 17, 58, 63, 72), it is probable that serum immunoglobulin G (IgG) antibodies to the O-specific polysaccharide will confer protection against *S. paratyphi* A.

The O-specific polysaccharides of serogroups A, B, and D sal-

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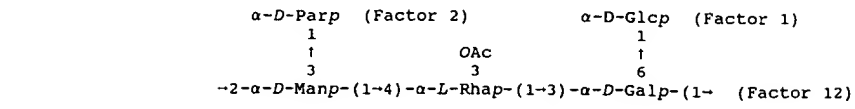

FIG. 1. Structure of the repeating unit of the O-specific polysaccharide of *S. paratyphi* A (1,2,12:a−) (23, 29).

monellae share a common backbone: →2-α-D-Man*p*-(1→4)-α-L-Rha*p*-(1→3)-α-D-Gal*p*-(1→ (10, 11, 16, 29). This structure is associated with the factor 12 specificity of the Kauffmann-White scheme (35). The serogroup specificity of *S. paratyphi* A is conferred by an α-3,6-dideoxyglucose (α-D-paratose) (factor 2) linked (1→3) to the mannose of the backbone (10–12, 29). The α-L-rhamnose of the backbone is partially O acetylated at C-3 (29) (Fig. 1). There is no antigen factor assignment for *O* acetyls of *S. paratyphi* A in the Kauffmann-White scheme.

In this study, the *S. paratyphi* A LPS was detoxified by treatment with acetic acid (O-SP) (15, 38, 72, 74) or with hydrazine (DeALPS) (26, 38, 65) and was bound to tetanus toxoid (TT) (15, 38, 57, 68, 72). The immunogenicity in mice and bactericidal activities of antibodies elicited by these conjugates and the importance of *O* acetyls are examined.

MATERIALS AND METHODS

Reagents. Anhydrous hydrazine, adipic acid dihydrazide (ADH), 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP), 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (EDAC), RNase, and DNase I were from Sigma Chemical Co., St. Louis, Mo.; 2,4,6-trinitrobenzenesulfonic acid (TNBS) and bicinchoninic acid were from Pierce Chemical Co., Rockford, Ill.; cyanogen bromide (CNBr) was from Eastman Kodak Co., New Haven, Conn.; proteinase K was from Boehringer Mannheim Biochemicals, Indianapolis, Ind.; acetic acid was from Mallinckrodt Specialty Chemicals Co., Paris, Ky.; Sephadex G-75, Sepharose CL-6B, Superose 12, and dextrans for calibration of molecular sizes were from Pharmacia LKB Biotechnology, Inc., Piscataway, N.J.; affinity-purified, alkaline-phosphatase-labeled goat antibodies to mouse Ig were from Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.; *Limulus polyphemus* amoebocyte lysate (LAL) was from Associates of Cape Cod, Woods Hole, Mass.; disodium 4-nitrophenyl phosphate hexahydrate was from Fluka, Ronkonkoma, N.Y.; pyrogen-free saline (PFS) and pyrogen-free water (PFW) were from Baxter Health Care Corporation, Deerfield, Ill.; phenol was from Fisher Scientific, Fair Lawn, N.J.; tryptic soy broth (TSB) was from Difco Inc, Detroit, Mich.; and triethylamine (TEA) was from Aldrich Chemical Co., Milwaukee, Wis. Rabbit anti-TT serum was donated by William Habig, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, Md.

Bacteria. *S. paratyphi* A NTP-6 was obtained from the blood culture of a patient in Kathmandu, Nepal (2). *S. typhimurium* (group B) TML (O:4, 12) was from Alison O'Brien, Uniformed Services University of the Health Sciences, Bethesda, Md. (72). Serogroups were identified by agglutination with grouping antiserum and were confirmed by the Laboratory of Microbiology, Clinical Center, National Institutes of Health.

LPS. LPS was purified from *S. paratyphi* A as described elsewhere (15, 38, 72, 74). Briefly, formalin-inactivated cells (200 g) were washed twice in PFS, suspended in PFW, and mixed with an equal volume of 90% phenol at 68°C for 30 min. The suspension was centrifuged at 7,300 × *g* at 10°C for 1 h. The aqueous layer was brought to 75% ethanol, and the precipitate was treated with DNase (50 mg) and RNase (50 mg). Proteinase K (200 mg) was added, and the suspension was dialyzed against Tris buffer overnight at 37°C and against PFW overnight at 3 to 8°C. The suspension was centrifuged at 64,000 × *g* at 10°C for 5 h and freeze-dried (weight, 1.5 g).

Detoxification of LPS by two methods. (i) Acid hydrolysis (15, 38, 72, 74). LPS (300 mg in 30 ml of 1% acetic acid [pH ~3]) was heated at 100°C for 1.5 h. The pH of the mixture was brought to 6.8 with 3.5 M NaOH, and the mixture was ultracentrifuged at 64,000 × *g* at 10°C for 5 h. The supernatant was freeze-dried, suspended in 5 ml of 0.2 M NaCl, and passed through a column (3 by 46 cm) of P-10 in PFW. The void volume fractions were pooled and freeze-dried. The powder, dissolved in PFW (20 mg/ml), was passed through a column (2.5 by 90) of Sephadex G-75, and the void volume fractions were pooled, freeze-dried, and designated O-SP. In another experiment, the powder (vide supra) was dissolved in PFW (20 mg/ml) and passed through a column (1.5 by 90 cm) of CL-6B Sepharose (Fig. 2). The first and second peaks were dialyzed against PFW, freeze-dried, and designated O-SP(HMW) and O-SP(LMW), respectively.

(ii) Hydrazinolysis (26). LPS (300 mg) was dried over P_2O_5 for 5 days, suspended in 30 ml of anhydrous hydrazine, and stirred at 37°C for 2 h. This solution was placed on ice, and cold acetone was added dropwise to a final concentration of ~90%. The resultant precipitate was removed by centrifugation at 15,000 × *g* at 10°C for 30 min, washed twice with cold acetone, dissolved in PFS (~5 mg/ml), and centrifuged at 64,000 × *g* at 10°C for 5 h. The supernatant was freeze-dried, dissolved in 0.2 M NaCl (5 ml), and passed through a column (3 by 46 cm) of P-10 in PFW. The void volume fractions were pooled, freeze-dried, and designated DeALPS. As with O-SP, DeALPS was passed through CL-6B Sepharose, and the void volume fractions were pooled, dialyzed, freeze-dried, and designated DeALPS(HMW).

Proteins. TT, lot GYA, from Pasteur Mérieux Serum et Vaccins, Lyon, France, was passed through S-300 Sephacryl column (15).

Derivatization of polysaccharide with ADH. The detoxified polysaccharides were derivatized by two methods. Method 1 was as follows. O-SP and DeALPS were derivatized with ADH as described elsewhere (15, 38, 57, 72). Polysaccharide (5 mg/ml in PFS) was brought to pH 10.5 to 11 with 0.1 M NaOH, and an equal amount of CNBr (1 g/ml of acetonitrile) was added. The reaction was carried out for 6 min on ice, and the pH was maintained at 10.5 to 11 with 0.1 M NaOH. An equal volume of 0.8 M ADH in 0.5 M $NaHCO_3$ was added, and the pH was adjusted to 8.5 with 0.1 M HCl. The reaction mixture was stirred at 4°C overnight and dialyzed against PFS at the same temperature for 24 h. The mixture was freeze-dried, reconstituted in 5 ml of PFW, and passed through a column (3 by 46 cm) of P-10 in PFW. The void volume fractions were pooled and freeze-dried. Method 2 was as follows. O-SP was activated with CDAP (37, 42). A 60-μl volume of CDAP (100 mg/ml of acetonitrile) was added to a solution of O-SP (2 ml, 10 mg of polysaccharide per ml of PFS) at room temperature. The pH was maintained at 5.8 to 6.0 for 30 s, and 60 μl of 0.2 M TEA was added to a pH of 7.0. The reaction was carried out for 2 min, and an equal volume of 0.8 M ADH in 0.5 M $NaHCO_3$ was added. This reaction was carried out for 2 h, and the pH was maintained at 8.0 to 8.5 with 0.1 N NaOH. The reaction mixture was dialyzed against PFS and passed through a column (3 by 46 cm) of P-10 in PFW. The void volume fractions were pooled, freeze-dried, and designated O-SPC.

Conjugation. ADH-derivatized polysaccharide (10 mg) was dissolved in PFS (2 ml). An equal weight of protein was added, and the pH was maintained at 5.1 to 5.5 with 0.1 M HCl. The reaction mixture was put on ice. EDAC was added to a final concentration of 0.05 M, and the pH was maintained at 5.1 to 5.5 for 4 h in 0.1 M HCl. The reaction mixtures were dialyzed against 0.2 M NaCl for 2 days with three changes of outer fluid and were passed through a column (1.5 by 90 cm) of Sepharose CL-6B in 0.2 M NaCl. The void volume fractions were stored at 3 to 8°C. The conjugates prepared with ADH as a spacer were designated O-SP(HMW)-TT, O-SP(LMW)-TT, O-SP-TT, DeALPS(HMW)-TT, DeALPS-TT, and O-SPC-TT_1.

In one preparation, O-SP was first activated by CDAP and 0.2 M TEA. An equal weight of TT was added (no ADH spacer), and the pH was maintained at 8.0 to 8.5 with 0.1 N NaOH for 2 h. The reaction mixture was passed through a column (1.5 by 90 cm) of CL-6B Sepharose in 0.2 M NaCl, and the void volume fractions were designated O-SPC-TT_2.

Analyses. The molecular sizes of LPS, O-SP, and DeALPS were estimated by gel filtration through Superose 12 in 0.2 M NaCl–0.001 M EDTA–0.01 M Tris–0.25% deoxycholic acid (pH 8.0), with dextrans as standards. The degrees of derivatization of O-SP and DeALPS with ADH were measured with TNBS (15). The amounts of protein were measured by using the bicinchoninic acid reagent with bovine serum albumin as a standard (26), and the amounts of hexose were measured by anthrone reaction with O-SP as a standard (34). LPS was detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (70). The amounts of *O* acetyl were measured by the Hestrin reaction with acetylcholine chloride as a standard (30).

^{13}C NMR. ^{13}C nuclear magnetic resonance spectroscopy (NMR) spectra of O-SP and DeALPS (25 mg of polysaccharide per ml of D_2O) was recorded on a Varian GN 300 spectrometer at room temperature with a 5-s decay between acquisition, and a 10-μs 90° pulse and approximately 50,000 free induction decay

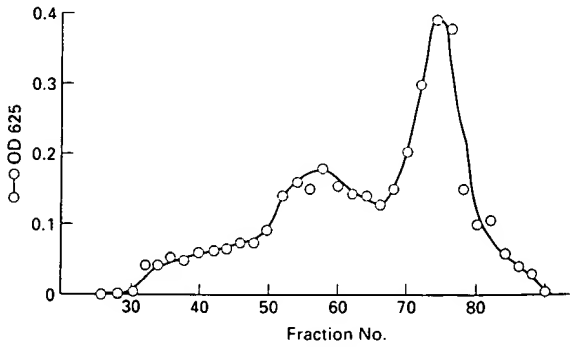

FIG. 2. Gel filtration profile of O-SP through a column (1.5 by 90 cm) of CL-6B Sepharose in 0.2 M NaCl. Fraction size, 5 ml; O-SP(HMW), fractions 30 to 48; O-SP(LMW), fractions 49 to 65. OD, optical density.

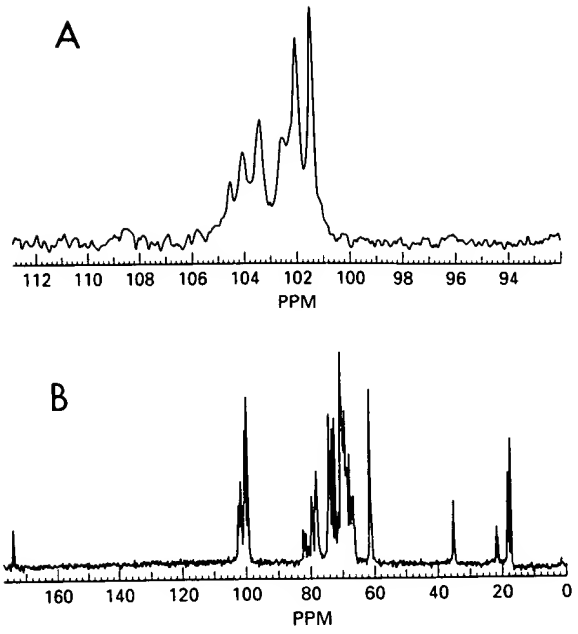

FIG. 3. ^{13}C NMR spectra of the O-SP of *S. paratyphi* A in D_2O with a General Electric GN300 spectrometer averaging ~42,000 scans at room temperature. (A) Region of anomeric carbon signals; (B) complete spectrum of carbonyl carbon from an acetamido group and methyl groups.

were averaged for each spectrum. Prior to Fourier transformation, a 3-Hz line broadening was applied and zero filled to 32,000 datum points.

Bioassays. Amounts of endotoxin were assayed by LAL and were expressed in endotoxin units (EU) related to the U.S. standard (32). Complement-mediated bactericidal activity was assayed against the NTP-6 strain (26, 38). Briefly, five-fold serum dilutions in 1% peptone were mixed with equal volumes of ~10^4 cells per ml in TSB supplemented with 5% rabbit serum as the source of complement. The mixture was incubated at 37°C for 1 h, 50 μl was spread onto TSB-agarose, and the plate was incubated overnight at 37°C. Titers were expressed as reciprocals of the highest dilution of serum that yielded 50% bactericidal activity. Controls included complement alone, sera from mice injected with saline, and hyperimmune sera without complement.

Immunization. Hyperimmune LPS antiserum was prepared by injecting adult female general-purpose mice from the NIH colony with heat-killed *S. paratyphi* A NTP-6 (15). For evaluation of immunogenicity, 5- to 6-week-old female general-purpose mice from the NIH colony were immunized subcutaneously once, twice, or three times at 14-day intervals with 2.5 μg of polysaccharide alone or as a conjugate. Mice from each experimental group of 10 were exsanguinated 7 days after each injection (38).

Serology. Double immunodiffusion was performed in 0.8% agarose in phosphate-buffered saline (PBS). LPS and TT antibody levels were determined by enzyme-linked immunosorbent assaying (ELISA) with Immulon 4 plates (Dynatech, Chantilly, Va.) (15). Each well was coated with 100 μl of 10 μg/ml of LPS or 20 μg/ml of protein in PBS. IgG and IgM anti-LPS levels were expressed in ELISA units with the hyperimmune serum, which was assigned a value of 100 Units, as a reference.

Statistical evaluation. Antibody levels are expressed as geometric means (GM). Levels that were less than the sensitivity of ELISA were assigned the value of one-half of that level. GM were compared by either the two-sided *t* test or the Wilcoxon test.

RESULTS

Characterization of LPS, DeALPS, and O-SP. ^{13}C NMR spectra (Fig. 3) for the anomeric carbons showed six signals (104.5, 104, 103.4, 102.5, 102, and 101.4 ppm) for O-SP (Fig. 3A) and five signals (104.5, 103.5, 102.5, 102, and 101.4 ppm) for DeALPS (not shown). The O-SP spectrum also had a signal at 174 ppm, which is characteristic of the carbonyl from an *O* acetyl group, and two signals (21.5 and 21 ppm), which is characteristic of methyl carbons of *O* acetyl substituents (Fig. 3B). These signals were not detected in the DeALPS spectrum. Each spectrum showed one signal (35 ppm) that was characteristic of the deoxy carbon of paratose and two signals (17.8 and 17 ppm) that were characteristic of the methyl carbons from the paratose and rhamnose. The *O*-acetyl contents of *S. paratyphi* A polysaccharides were as follows (in percent moles of *O* acetyl per mole repeat unit): LPS, 0.82; O-SP, 0.83; O-SP-AH, 0.59; O-SPC-AH, 0.80; DeALPS, 0.09.

Figure 4 shows the ladder pattern of LPS on SDS-PAGE. The sensitivity of this assay is ~1 μg of LPS. At 10 μg, O-SP showed a faint smear at the top of the gel and no ladder formation. In contrast, DeALPS showed a faint ladder at 10 μg. By LAL assaying, the LPS had 7×10^3 EU per μg and O-SP and DeALPS had 0.70 EU per μg, each representing an ~10,000-fold reduction. LPS, DeALPS, and O-SP each had ~1.5% protein and nucleic acids.

High-performance liquid chromatography of LPS and O-SP showed four peaks. LPS had K_ds of 0.11 (130 kDa), 0.34 (35 kDa), 0.49 (14.3 kDa), and 0.59 (8.3 kDa). O-SP had K_ds of 0.13 (122 kDa), 0.41 (23.4 kDa), 0.56 (9.6 kDa), and 0.64 (6.0 kDa). DeALPS had K_ds of 0.12 (128 kDa), 0.37 (31 kDa), and 0.53 (11.6 kDa). Passage through CL-6B Sepharose yielded two fractions with molecular masses for O-SP(HMW) of 122 kDa and for O-SP(LMW) of 23.4 kDa and only one fraction for DeALPS, which was denoted DeALPS(HMW), with a molecular mass of 130 kDa.

Double immunodiffusion of LPS, O-SP, and DeALPS with the hyperimmune serum showed partial identity reactions, with spurs extended from the LPS over O-SP, which had a spur over DeALPS (Fig. 5A).

Characterization of conjugates. After treatment of O-SP with CNBr, amounts of *O* acetyls decreased from 82 to 59% mol/mol. Activation by CDAP and then derivatization with ADH, in contrast, did not reduce the *O*-acetyl content. The adipic hydrazide (AH) content of O-SP activated with CNBr (method 1) was 3.0%, and that with CDAP (method 2) was 2.9% (Table 2). For DeALPS activated with CNBr, the AH content was 1.8%. O-SP-AH formed a line of identity with O-SP when reacting with the hyperimmune serum in double immunodiffusion (Fig. 5B). Similarly, DeALPS yielded a line of identity with its AH derivative (not shown).

All conjugates emerged as a single peak in the void volume of the CL-6B Sepharose column, and these fractions and the pool gave identical lines of precipitation with the *S. paratyphi* A hyperimmune and rabbit anti-TT sera (Fig. 5C). Table 2 shows

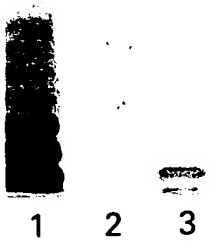

FIG. 4. Silver-stained 0.1% SDS–14% PAGE pattern of *S. paratyphi* A LPS and polysaccharides. Lanes: 1, 1 μg of LPS; 2, 10 μg of O-SP; 3, 10 μg of DeALPS.

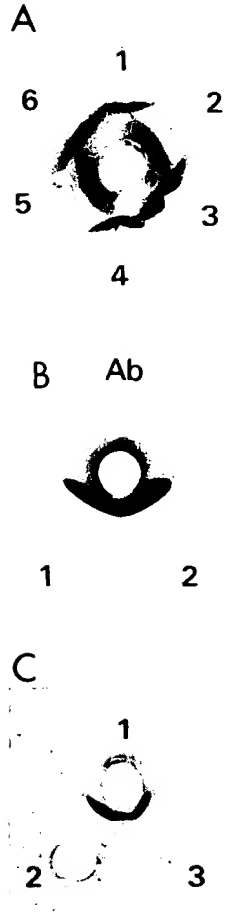

FIG. 5. Double immunodiffusion in 1% agarose. (A) Center well, hyperimmune *S. paratyphi* A antiserum; surrounding wells, antigens (500 μg/ml): wells 1 and 4, LPS; wells 2 and 5, DeALPS; wells 3 and 6, O-SP. (B) Top, hyperimmune *S. paratyphi* A antiserum (antigens at 1 mg/ml): well 1, O-SP; well 2, O-SP-AH. (C) Well 1, O-SP-TT (1 mg saccharide per ml); well 2, hyperimmune *S. paratyphi* A antiserum; well 3, anti-tetanus toxoid.

that the polysaccharide-to-protein ratios of the conjugates ranged between 0.95 and 2.05. The yield of saccharide in the conjugates was between 22 and 75%, with the lower-molecular-weight O-SP(LMW)-TT having the lowest yield.

LPS antibodies in mice (Table 3). Saline, DeALPS, O-SP, DeALPS(HMW)-TT, and DeALPS-TT did not elicit detectable LPS antibodies after any injection.

None of the conjugates listed in Table 3 elicited anti-LPS after one injection. After the second and third injections, all conjugates except O-SP(LMW)-TT elicited low levels of IgM antibodies.

Both conjugates O-SP(HMW)-TT and O-SP-TT, prepared with CNBr-activated O-SP, elicited IgG antibodies after two injections. Each conjugate elicited a booster response after the third injection; however, there was no significant difference between the IgG levels of anti-LPS (3.01 versus 2.05; not significant [NS]).

Conjugates prepared with CDAP-activated O-SP (O-SPC-TT_1 and O-SPC-TT_2) showed similar antibody responses after two or three injections. The antibody levels elicited by O-SPC-TT_1 (with spacer) and O-SPC-TT_2 (without spacer) were similar (2.37 versus 1.72 [NS]).

Although the level was slightly higher for O-SP(HMW)-TT, there was no statistically significant difference in LPS antibody levels after the third injection of these four conjugates (3.01 versus 2.05, 2.37, and 1.72 [NS]).

Bactericidal antibodies (Table 4). Neither saline, O-SP, nor DeALPS elicited bactericidal antibodies to *S. paratyphi* A (not shown). Sera with high titers, as measured by ELISA, from individual mice injected with conjugates had bactericidal activity against *S. paratyphi* A. These sera had no effect on *S. typhimurium* TML (not shown). Sera from mice injected with DeALPS(HMW)-TT and DeALPS-TT, with no detectable anti-LPS by ELISA, did not have bactericidal activity.

Protein antibodies (Table 5). All conjugates, including those with DeALPS, elicited TT antibodies with booster responses. Mice injected with O-SPC-TT_2, which does not contain a spacer, elicited the highest anti-TT levels after one or two injections ($P < 0.005$).

DISCUSSION

Enteric fever is a systemic infection caused by *S. typhi* and salmonellae of groups A to D (9, 21, 41, 55–57). There is evidence that the surface polysaccharides of salmonellae are protective antigens, and we proposed that a critical level of serum IgG anti-LPS could initiate complement-mediated lysis of these pathogens as they enter the intestine (13, 26, 55–57, 65, 68). Vaccination with the surface polysaccharide of *S. typhi* (Vi) prevents typhoid fever (2, 36). The immune moiety elicited by Vi, like that elicited by other capsular polysaccharide-based vaccines, is mainly serum antibody (55–57). The surface polysaccharide of *S. paratyphi* A alone is not immunogenic in mice, most likely because of its comparatively low M_r (51, 66). There is experimental evidence that serum antibodies to the O-specific polysaccharides of group B and D salmonellae, whether actively induced or passively administered, confer protection to mice (12, 16, 55, 56, 63, 72). The failure of bacterial vaccines, such as TAB, to confer protection against group A and B salmonellae may be likened to the failure of parenterally injected inactivated gram-negative bacteria to induce high lev-

TABLE 1. Description of *S. paratyphi* A conjugates

Conjugate	Description (reference)
O-SP	Acetic acid-detoxified LPS passed through G-75 Sephadex (15, 74)
DeALPS	Hydrazine-detoxified LPS passed through G-75 Sephadex (26)
O-SP(HMW)-TT and DeALPS(HMW)-TT	First peak of O-SP or DeALPS from Sepharose CL-6B (HMW, high molecular weight), activated with CNBr, derivatized with ADH, and bound to TT
O-SP(LMW)-TT	Second peak of O-SP from Sepharose CL-6B (LMW, low molecular weight), activated with CNBr, derivatized with ADH, and bound to TT
O-SP-TT and DeALPS-TT	O-SP or DeALPS, activated with CNBr, derivatized with ADH, and bound to TT
O-SPC-TT_1	O-SP activated with CDAP, derivatized with ADH, and bound to TT
O-SPC-TT_2	O-SP activated with CDAP and bound to TT

TABLE 2. Composition of *S. paratyphi* A polysaccharide-protein conjugates

Conjugate	% AH/ saccharide	Saccharide/ protein	Yield (%)[a]
O-SP-TT	3.0	2.05	34.1
O-SP(HMW)-TT	3.0	1.34	74.3
O-SP(LMW)-TT	3.0	0.99	22.2
O-SPC-TT_1	2.9	1.17	31.5
O-SPC-TT_2	NA[b]	0.95	33.9
DeALPS(HMW)-TT	1.8	1.35	71.6
DeALPS-TT	1.8	1.84	62.8

[a] Based on the weight of the saccharide.
[b] NA, Not applicable.

els IgG anti-LPS antibodies (6, 18, 56, 61). Another explanation for the apparent failure of the nontyphoidal components might have been gradual hydrolysis of *O* acetyls of the LPSs in TAB vaccine.

Subcutaneous injection of mice with comparatively low doses in saline of our *S. paratyphi* A conjugates elicited bactericidal antibodies specific for group A but not group B salmonellae (72). This indicates that our *S. paratyphi* A conjugates elicited bactericidal antibodies directed toward group-specific antigen (factor 2) conferred by paratose and is consistent with proposed structures of the group A, B, and D O-specific polysaccharides of salmonellae (10, 11, 17).

O acetyls are essential for eliciting anti-LPS with bactericidal activity against *S. paratyphi* A. Treatment of *S. paratyphi* A LPS with acetic acid retained 80% of the *O* acetyls. Hydrazinolysis, a clinically acceptable method for detoxification of LPS (26, 38), removed the *O* acetyls; conjugates prepared with the *O*-acetyl-negative DeALPS did not elicit anti-LPS with bactericidal activity. *O* acetyls are essential for the immunogenicity of other medically important polysaccharides including those of *Streptococcus pneumoniae* type 1, *S. typhi* (Vi), *Staphylococcus aureus* type 8, and group B and C *Neisseria meningitidis* (5, 50, 67).

In agreement with the report that the C-3 of rhamnose of the O-specific polysaccharide of *S. paratyphi* A is partially O acetylated, we found a molar ratio of 0.82 *O*-acetyl per repeat unit (29). However, we detected two acetamido signals in the ^{13}C NMR spectrum. The O-specific polysaccharide of *S. typhimurium* (group B) is 2-O acetylated at the dideoxygalactose, abequose, conferring the specificity that is defined as factor 5 (10, 11, 23). Carlin et al. reported that a murine monoclonal IgA to factor 5 had a low level of protective activity against *S. typhimurium* (12). However, it cannot be concluded that the O acetyl may not have important biologic activity for group B salmonellae, because IgA may not exert protection in bloodborne infection. One explanation for the failure of de-O-acetylated O-specific polysaccharide of *S. paratyphi* A to induce bactericidal antibodies is that paratose is partially O acetylated in the native saccharide. Since it has been presumed that immunity to nontyphoidal salmonellae is largely group specific and that the dideoxy sugars are essential for this specificity (10, 11, 17), we intend to investigate further the location(s) and immunologic roles of *O* acetyls in O-specific polysaccharides of groups A, B, and D.

CDAP has advantages over CNBr for synthesis of *S. paratyphi* A conjugates (37, 42). First, activation by CDAP occurred at neutral pH and did not reduce *O* acetyls on the O-specific polysaccharide. Second, CDAP-activated polysaccharide could be bound directly to TT without adding ADH as a spacer (42). O-SPC-TT_2, which had TT directly bound to O-SP without a spacer, elicited the highest level of TT antibodies ($P <$

TABLE 3. Immunoglobulin class composition of serum LPS antibodies elicited by *S. paratyphi* A-TT conjugates in mice[a]

Immunogen	GM antibody level (ELISA U)					
	One injection		Two injections		Three injections	
	IgG	IgM	IgG	IgM	IgG	IgM
O-SP(HMW)-TT	0.03	0.03	0.18	0.14	3.01	0.41
O-SP(LMW)-TT	0.05	0.05	0.07	0.04	0.19	0.03
O-SP-TT	0.04	0.03	0.35	0.26	2.05	0.28
O-SP-TT_1	0.02	0.02	0.30	0.23	2.37	0.33
O-SPC-TT_2	0.02	0.04	0.26	0.17	1.72	0.31

[a] 5- to 6-week-old female mice were injected subcutaneously once, twice, or three times at 14-day intervals with 2.5 μg of saccharide alone or as a conjugate. Mice from each group (n = 10) were exsanguinated 7 days after each injection, and anti-LPS was measured by ELISA (15, 38). IgM and IgG hyperimmune sera were from mice injected multiple times, with heat-killed *S. paratyphi* A used as a standard and were assigned a titer value of 100 U each (15, 38). IgG GMs were 0.18, 0.35, 0.30, and 0. 26 versus post-first injection ($P < 0.001$); 3.01, 2.05, 2.37, and 1.72 versus second injection ($P < 0.001$); and 3.01 versus 2.05, 2.37, and 1.72 (NS).

TABLE 4. Complement-dependent bactericidal activity of serum LPS antibodies elicited in mice by immunization with heat-killed *S. paratyphi* A or O-specific polysaccharide-protein conjugates

Immunogen	Anti-LPS titer (ELISA U)		Bactericidal titer[a]
	IgG	IgM	
S. paratyphi A[b]	100.0	100.0	32,000
O-SP-TT	7.73	0.54	160
O-SP-TT	5.89	0.10	160
O-SP(HMW)-TT	7.38	0.76	160
O-SP(HMW)-TT	6.14	0.90	160
O-SPC-TT_1	8.49	0.26	>640
O-SPC-TT_1	9.93	0.42	1,280
O-SPC-TT_2	7.51	0.70	320
O-SPC-TT_2	6.97	0.39	640
DeALPS(HMW)-TT	0.20	0.04	0
DeALPS-TT	0.01	0.03	0

[a] Expressed as the highest serum dilution yielding 50% killing.
[b] Pooled sera from mice injected i.v. with heat-killed *S. paratyphi* A (38, 72). The correlation coefficients between IgG or IgM with bactericidal titer are 0.7 (P = 0.015) and 0.02, respectively. Sera were from individual mice taken after the third injection of the conjugate. Dilutions of sera were mixed with equal volumes of ~10^4 *S. paratyphi* A per ml and complement, incubated at 37°C, and counted as described in Materials and Methods.

TABLE 5. Serum TT antibodies in mice injected with *S. paratyphi* A-TT conjugates

Immunogen	GM antibody levels[a]		
	One injection	Two injections	Three injections
Saline	ND	ND	0.06
O-SP(HMW)-TT	0.18	2.25	11.03
O-SP(LMW)-TT	0.38	10.01	13.94
O-SP-TT	0.36	6.83	8.31
O-SPC-TT_1	0.24	3.55	14.91
O-SPC-TT_2	0.77	26.04	62.02
DeALPS(HMW)-TT	0.13	3.72	4.91
DeALPS-TT	0.12	1.96	4.90

[a] TT antibody levels expressed in ELISA units with a high titer responder as the reference and assigned a value of 100 U (38). ND, not done.

0.005), although the lowest level of anti-LPS (NS). O-SPC-TT_2 bound mostly to lysines on the protein, while carbodiimide condensation was used to activate glutamic or aspartic acids to form other conjugates. Since both the yields and immunogenicity for the saccharide component of O-SPC-TT_2 were satisfactory for conjugates prepared with CDAP, we plan to explore further the uses of this and similar reagents.

The immunogenicity of the saccharide component of conjugates is related to its molecular size. Similarly to what was observed for Vi and pneumococcus type 4, *S. paratyphi* A conjugates prepared with low-molecular-weight O-SP did not elicit detectable LPS antibodies (13, 51, 56–58, 63, 66, 67).

S. paratyphi A is a pathogen for humans only, and we propose that conjugate-induced bactericidal antibodies provide a correlate for immunity, as shown for respiratory gram-negative pathogens (22, 56–58, 65, 67). Vaccination of young mice with our *S. paratyphi* A conjugates by a clinically relevant scheme induced bactericidal antibodies.

In summary, *S. paratyphi* A conjugates elicited serum IgG antibodies in mice with bactericidal activity. Because the *O* acetyls are essential, the O-specific polysaccharide by acid hydrolysis, bound to proteins with CDAP, provides a useful method for preparing *S. paratyphi* A conjugates. We feel that there is sufficient information from licensed vaccines, including Vi (58), and from investigational *S. typhimurium* and *Shigella* O-polysaccharide conjugates (57, 58, 63, 72) to evaluate clinically our *S. paratyphi* A conjugates; if effective, conjugates against group B, C, and D salmonellae can be predicted to confer protection against these other causes of enteric fever.

ACKNOWLEDGMENTS

We are grateful to Vee Gill and the staff of the Microbiology Branch, Clinical Center, NIH, for their assistance. James C. Mond, Uniformed Services University of the Health Sciences, Bethesda, Md., and Andrew Lees, Virion Systems Inc, Rockville, Md., provided helpful advice about their experience with CDAP.

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Editor: J. R. McGhee